Infrequent Mutation in the *BRCA2* Gene in Esophageal Squamous Cell Carcinoma

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ABSTRACT

Purpose: **Previous studies have shown a high rate of allelic loss in esophageal squamous cell carcinoma (ESCC) in the vicinity of the *BRCA2* gene. We aimed to assess whether the tumor suppressor gene *BRCA2* was the inactivation target for allelic loss observed on chromosome 13q in ESCC.**

Experimental Design: **We examined the entire coding sequence of the *BRCA2* gene for mutations using single-strand conformation polymorphism analysis and DNA sequencing in 56 ESCC patients from Shanxi, China.**

Results: **Eight mutations were identified in 5 patients (9%), including 3 with germ-line mutations and 2 with only somatic mutations. However, all but 1 of the mutations were missense or silent changes and of unknown significance. Evidence for potential biallelic inactivation was seen in only 4 (7%) cases.**

Conclusions: BRCA2 **mutations occur in ESCC but are infrequent and of unknown consequence. The putative target tumor suppressor gene corresponding to the high rate of chromosome 13q allelic loss remains unknown.**

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INTRODUCTION

The *BRCA2* gene is located on chromosome 13q (1). Alterations in the *BRCA2* gene result in increased risk of breast cancer in both women and men, and a moderately increased risk for a variety of other cancers, including carcinomas of the ovary, pancreas, prostate, colon, and liver (2–7). Thus far only infrequent alterations in *BRCA2* have been reported in ESCC (8).[3] Not surprisingly, few studies have reported mutation frequencies for all of the coding exons of *BRCA2* because of its large size. *BRCA2* is thought to be involved in double-strand DNA break repair (9, 10). Several studies have demonstrated that BRCA2 and BRCA1 bind to Rad51, a protein involved in maintaining the integrity of the genome. Rad51 also physically associates with the TP53 tumor suppressor protein. Physical and functional interactions of *BRCA2* with these key components of cell cycle control and DNA repair pathways suggest that it likely participates with them in some way to maintain genomic integrity (11). This association is additionally supported by the fact that somatic mutations of *TP53* are commonly seen with germline mutations of *BRCA1* and *BRCA2* in breast/ovarian cancer (12, 13).

Esophageal cancer is a very common disease in many areas of China, especially in Shanxi Province (14). In previous studies in Shanxi Province, China, we found frequent LOH on chromosome 13 (15, 16), including chromosome 13q12 where *BRCA2* is located (15–17). In the present study we characterized genetic alterations in *BRCA2* in ESCC patients by screening the entire *BRCA2* gene for mutations using SSCP analysis and DNA sequencing in 56 ESCC patients examined previously for both *TP53* mutations and LOH on chromosome 13q (18, 19).

MATERIALS AND METHODS

Patient Selection. Patients presenting in 1995 and 1996 to the Shanxi Cancer Hospital in Taiyuan, Shanxi Province, People's Republic of China, who were diagnosed with ESCC and considered candidates for curative surgical resection, were identified and recruited to participate in this study. The study was approved by the Institutional Review Boards of the Shanxi Cancer Hospital and the United States National Cancer Institute. A total of 56 patients with ESCC were selected who had a histological diagnosis of ESCC confirmed by pathologists at both the Shanxi Cancer Hospital and the National Cancer Institute. None of the patients had prior therapy, and Shanxi was the ancestral home for all of the patients.

After obtaining informed consent, patients were interviewed to obtain information on demographic and cancer lifestyle risk factors, and a detailed family history of cancer. A total

[3] The abbreviations used are: ESCC, esophageal squamous cell carcinoma; SSCP, single-strand conformation polymorphism; LOH, loss of heterozygosity; UGI, upper gastrointestinal.

of 56 ESCC patients, including 34 males and 22 females, were evaluated. Details on these ESCC patients have been described previously (19). All of the patients were previously evaluated for allelic loss on 13q, including D13S260 and D13S267, which flank *BRCA2* (15–17) and mutations in *TP53* (exons 4 to 9; Ref. 18). The frequencies of LOH on D13S260 and D13S267 were 57% (17 of 30 informative cases) and 83% (33 of 40 informative cases), respectively (17). Mutations in *TP53* were found in 77%, and intragenic allelic loss was observed in 76% (18).

Biological Specimen Collection and Processing. Venous blood (10 ml) was taken from each patient before surgery, and genomic DNA was extracted and purified. Tumor tissue obtained during surgery was fixed in ethanol and embedded in paraffin.

Laser Microdissection and Extraction of DNA. Tumor cells were microdissected under light microscopic visualization using methods described previously (20).

PCR and SSCP Analysis. Mutations in all 26 coding exons of the *BRCA2* gene were screened by PCR-SSCP. The 57 pairs of PCR primers used to cover all of the intron/exon boundaries are listed in Table 1. DNA extracted from tumor cells was microdissected from the resection specimen, and genomic DNA extracted from venous blood was used for each patient. PCR reactions and SSCP analyses were conducted using methods described previously (19) except the annealing temperature was adjusted to 55–60°C.

DNA Sequencing. DNA sequencing was performed using methods described previously (19). All of the mutations were confirmed by repeating the procedures outlined above. Subcloning was performed in 1 case (SHE247) with the TOPO Cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Statistical Analysis. All of the statistical analyses were performed using Statistical Analysis Systems (SAS; SAS Corp., Cary, NC). Associations were tested using Fisher's exact test. All P were two-sided and considered statistically significant if $P < 0.05$.

RESULTS

Screening the entire coding region of the *BRCA2* gene in tumor and blood DNA of 56 ESCC patients identified 8 mutations in 5 cases (5 of 56; 9%). Three cases had germ-line mutations, whereas 2 had only somatic mutations. These mutations are listed in Table 2*a*, and examples are shown in Figs. 1 and 2. Demographic characteristics and previously determined genetic alterations of *TP53*, and LOH on D13S260 and D13S267 for these 5 cases are listed in Table 2*b*. No significant association was seen between alterations (mutations or intragenic allelic loss) in *BRCA2* and *TP53* (data not shown).

Allelic Loss at Polymorphic Sites in *BRCA2*. SSCP analysis of *BRCA2* exons 2–27 performed in this search for mutations in ESCC samples revealed bandshifts in some samples in exons 2, 10 (primer 10.3), and 11 (primer 11.7; Fig. 3). Direct sequencing of the genomic DNA/PCR products of these exons after SSCP showed the presence of three polymorphic sites (203G>A, N372H, and K1132K) reported previously in the Breast Cancer Information Core database.[4] The frequency of allelic loss in tumor DNA at these three polymorphic sites was 20% (10 of 51), 81% (13 of 16), and 64% (16 of 25) for 203G>A, N372H, and K1132K, respectively. Forty-six percent of ESCC cases (26 of 56) were found to have intragenic allelic loss at one or more of these polymorphic sites, including 16 with one, 7 with two, and 3 with loss at all three of the sites. Ten cases lost a wild-type allele at 203G>A; 5 cases lost a wild-type allele and 8 lost a polymorphic allele at N372H; and 8 lost a wild-type allele and 8 lost a polymorphic allele at K1132K (for example, see Fig. 3 for N372H).

Potential Biallelic Inactivation of *BRCA2*. We found evidence for potential biallelic inactivation of *BRCA2* in 4 of 56 (7%) cases (Table 2*a*). Two cases (SHE138 and SHE437) had a germ-line mutation in one allele and LOH in the other (wild-type) allele. A third case (SHE360) had a germ-line mutation in one allele (at codon 315) and LOH near the mutation position (at codon 372), but we could not determine whether the LOH was in the wild-type or mutant allele. A fourth case (SHE247) had two mutations (one missense and one frameshift) in different exons, but we do not know if these mutations occurred on different alleles. The fifth case (SHE150) also had two mutations, but because one mutation was silent and no other alterations were identified, it is unlikely that biallelic inactivation occurred. In addition, 10 cases without mutation had intragenic allelic loss at either two ($n = 7$) or all three ($n = 3$) of the polymorphic sites (data not shown). While it is possible that these losses occurred in different alleles, it seems more likely that these findings were the result of a single large allelic loss rather than multiple discrete events that occurred on different alleles.

Genetic Alterations of *BRCA2* and LOH at D13S260 and D13S267. The number of cases with a *BRCA2* mutation was too small for meaningful comparison with LOH at microsatellite markers D13S260 or D13S267; however, LOH at D13S267 was significantly associated with allelic loss of at least one of the polymorphisms within *BRCA2* ($P = 0.004$). Furthermore, among the 36 cases informative for both D13S267 and *BRCA2*, D13S267 showed LOH for all 20 cases with an intragenic *BRCA2* allelic loss (sensitivity = 20 of 20 = 100%, specificity = 6 of 16 = 38%). Twenty of the 30 cases with LOH at D13S267 were subsequently found to have intragenic allelic loss in *BRCA2* (positive predictive value = 67%). No significant association between LOH at D13S260 and loss at these three polymorphic sites was seen (data not shown).

Genetic Alterations of *BRCA2* and Family History. All 3 cases with germ-line mutations had a positive family history of UGI cancer. The frequency of *BRCA2* mutations was somewhat higher in patients with a family history of UGI cancer (12%) compared with patients without such a family history (5%), but this difference was not significant ($P = 0.36$). Also, there was a slightly higher frequency of allelic loss (53%, 18 of 34) at polymorphic sites in patients with a family history of UGI

[4] Internet address: http://www.nchgr.nih.gov/Intramural_research/Lab_transfer/BIC.

Table 1 Sequence of primers used for PCR-SSCP analysis of *BRCA2*

Exon[a]	Sense primer (5′–3′)	Antisense primer (5′–3′)
2	CTCAGTCACATAATAAGGAATGC	CAACACTGTGACGTACTGGGT
3	CAAATTTGTCTGTCACTGGTTA	CTAAATTCCTAGTTTGTAGTTC
4	ACACTTCCAAAGAATGCAAAT	TCTTCCTACAGGCTCTTAG
5	ATATCTAAAAGTAGTATTCCAACA	AAACTCCCACATACCACTGG
6	CTACAATGTACATGTAACAC	AATCTCAGGGCAAAGGTATAAC
7	CGTTAAGTGAAATAAAGAGTGAATGA	TAACAGAATTATTAGATGACAATT
8	GTGTCATGTAATTCAAATAGTAGATGT	AATGTAAGATAAATAATTTAACAAGG
9	TACTACTATATGTGCATTGAGA	ACAGAGCAAGACTCCACCT
10.1	TAATGTGCTTCTGTTTTATAC	ACATTCATCAGCGTTTGCTTC
10.2	CAAAGACCACATTGGAAAGTC	GATCAGTATCATTTGGTTCCAC
10.3	AAGCAAACGCTGATGAATGTG	TGGTCACATGAAGAAATATGC
10.4	CAGGTCTAAATGGAGCCCAG	GAGAAGTTCCAGATATTGCC
10.5	AAGCCTCTGAAAGTGGACTG	GCAAATGTAAGTGGTGCTTC
11	AAGTGAAAGACATATTTACAGACAG	TATGAAGCTTCCCTATACT
11.1	GATGGTACTTTAATTTGTCAC	CAAGATCCTGAGAGATTACTG
11.2	GCTCTTTTGGGACAATTCTG	ATAAAAGATTTTCTGGGATTG
11.3	TGGAATACAGTGATACTGAC	TTTTCAGGTGGCAACAGCTC
11.4	CCCATGGAAAAGAATCAAGATG	GTTCCTTAGTATTCCTAAAGC
11.5	TGTCTTCCAAGTAGCTAATG	CTGTGATTTGAAATTGGACC
11.6	ACATGAACAAATGGGCAGGAC	TGGTTTGAATTAAAATCCTGC
11.7	GTCATATAACCCCTCAGATG	CTGTACCTTCAAATTGCTTGC
11.8	CGATTGGTCAGGTAGACAGC	CTCTGCAGAAGTTTCCTCAC
11.9	TGTTTCTACTGAAGCTCTGC	GTTATCTTCATTTTCAGTATTTCTC
11.10	TTGAAATGACTACTGGCAC	CCTTCATAAACTGGCCAGATAAT
11.11	TGTCTTAAATTATCTGGCCAG	AAATGACTCTTTGGCGACAC
11.12	AGATTTTGAGACTTCTGATAC	TCCAGTACCAACTGGGACAC
11.13	TGGACATTCTAAGTTATGAGG	ATTTCACTAGTACCTTGCTCTTTT
11.14	TGATGAAAAAGAGCAGGTAC	ACAAGGTTTTTATCATTATTG
11.15	CTGCCCCAAAGTGTAAAGAAAT	AATGACTGAATAAGGGGACTGAT
11.16	TCCTGCAACTTGTTACAC	GATTTTTGTCATTTTCAGC
11.17	AACCAGAAAGAATAAATACT	CCTCAACGCAAATATCTTCAT
11.18	TTCCAAAGTAATATCCAATGTA	ATTTTTGATTTATTCTCGTTGTT
11.19	AAGTGAAAGACATATTTACAGACAG	TATGAAGCTTCCCTATACT
11.20	CACCTTGTGATGTTAGTTTG	TTGGGATATTAAATGTTCTGGAGTA
11.21	TACTCCAGAACATTTAATATCCCAA	CGTAGGTGTGAATAGTGAAGAC
11.22	GTCTTCACTATTCACCTACG	AGTGAGACTTTGGTTCCTAAT
11.23	TTCAACAAGACAAACAACAGT	GTCAGTTCATCATCTTCCATAAA
11.24	CTTACTCCAAAGATTCAGAAAACTAC	AGCATACCAAGTCTACTGAATAAAC
12	AAAATGGTCTATAGACTTTTGAG	ACCTATAGAGGGAGAACAGAT
13	ACAGTAACATGGATATTCTCTTA	AAACGAGACTTTTCTCATACTG
14.1	ATAAACTTATATATTTTCTCCC	AGGTGGAACAAAGACTTTGGT
14.2	TGAGACACTTGATTACATCAG	ATATCTAACTGAAAGGCAAA
15	ATTTAATTACAAGTCTTCAGAATG	ATAAAAGCCATCAGTATTGTAG
16	TTTATTGTGTGATACATGTTTACT	AAAGAGGGATGAGGGAATAC
17	GTTGAATTCAGTTACATCCTAT	ATAGGATGATACTGAATTCAAC
18	CTTGTTTAAACAGTGGAATTCTA	TAACTGAATCAATGACTGAT
19	GAATTGAATACATATTTAACTACTA	CCATCTCAAACAAACAAACAAAT
20	CACTGTGCCTGGCCTGATAC	AGTCTCTAAGACTTTGTTCTC
21	TATGCTTGGTTCTTTAGTTTTAG	CTCACCTTGAATAATCATCAAG
22	GTTCTGATTGCTTTTTATTCC	AGTAAGGTCATTTTTTAAGTTAAT
23	TTTAAATGATAATGACTTCTTCC	TCCATAAACTAACAAGCACTTAT
24	TTTATGGAATCTCCATATGTTGA	CTGGTAGCTCCAACTAATCTA
25	CTTAAAATTCATCTAACACATCTA	AAAAATACCAAAATGTGTGGTGA
26	ACATAAATATGTGGGTTTGCAAT	ACGATGGCCTCCATATATACT
27.1	GAGACTGTGTGTAATTATTTGCGT	GGTAAAGGCAGTCTACTCAAG
27.2	AGAGAAGAGCCTTGGATTTCT	TGGGTATTTATCAATGCAAGT
27.3	TCTTTTGTCTGGTTCAACAGG	AAGCGTCAATAATTTATTGTC

[a] Total $n = 57$.

cancer compared with patients without such a family history (36%, 8 of 22; $P = 0.28$).

DISCUSSION

Somatic mutations in *BRCA2* are very rare in breast cancer and other tumors (4–7, 21). Only one previous study has reported testing all of the coding exons in *BRCA2* in ESCC, and no mutations were detected in those Japanese patients (8). To our knowledge, our report is the first to identify germ-line or somatic mutations in *BRCA2* in ESCC patients. In the present study of 56 ESCC patients from a high-risk population in China, we found that 5 patients (9%) had 8 *BRCA2* mutations. How-

Table 2 Genetic changes and demographics for patients with *BRCA2* alterations

A. *BRCA2* genetic alterations in 5 of 56 ESCC patients

	Mutation						Allelic loss		Biallelic alterations
			BRCA2 mutation				Allelic loss		
Patient ID	Mutation	Exon	Codon/nucleotide	Base change	Amino acid change	Designation/ mutation type	Presence of LOH	Wild-type LOH	Evidence for biallelic alterations
SHE138	Germline	4	118/581	G→A CGC→CAC	Arg→His	R118H/missense	Yes	Yes	Yes
	Somatic	11	1682/5274	T→C AGT→AGC	Ser→Ser	S1682S[a]/silent	No	—	
SHE150	Somatic	11	1338/4242	C→T GGC→GGT	Gly→Gly	G1338G[a]/silent	No	—	No
	Somatic	11	1988/6190	G→A GTA→ATA	Val→Ile	V19881[a]/missense	No	—	
SHE247	Somatic	3	25–26/after 303	insertion 7bp TTAGGA(ccaatga) CCAATA	stop codon 30	303ins7[a]/frame shift	No	—	Possible
	Somatic	20	2842/8752	C→T CGC→TGC	Arg→Cys	R2842C[a]/missense	No	—	
SHE360	Germline	10	315/1171	T→A TGT→AGT	Cys→Ser	C315S/missense	Yes	Unknown	Possible
SHE437	Germline	27	3300/10126	C→T CCA→TCA	Pro→Ser	P3300S[a]/missense	Yes	Yes	Yes

B. Demographics and results of TP53 mutation and microsatellite marker LOH testing in ESCC patients with *BRCA2* mutations

Patient ID	Age/sex	Family history of cancer[b]	*TP53* mutation in exons 4–9	Intragenic allelic loss in R72P of *TP53*[c]	LOH at D13S260/267
SHE 138	55/F	EC (mother)	12bp del (codon 174)	Retention	Loss/homozygous
SHE 150	57/M	EC (mother), cervical cancer (paternal aunt)	No	Homozygous (Pro/Pro)	Retention/retention
SHE247	45/M	No	2bp del (codon 69)	Homozygous (Arg/Arg)	Homozygous/homozygous
SHE360	55/M	EC (father), BC (brother)	No	Loss (Arg allele)	Loss/loss
SHE437	47/F	2 EC (father and mother)	18bp del (Codon 134)	Retention	Loss/loss

[a] Not reported in the BIC as of February 2001.4
[b] Includes complete family history of cancer in first, second, and third degree relatives; EC, esophageal cancer; BC, body of stomach cancer.
[c] Polymorphism at codon 72, Arg→Pro, in exon 4 of *TP53*.

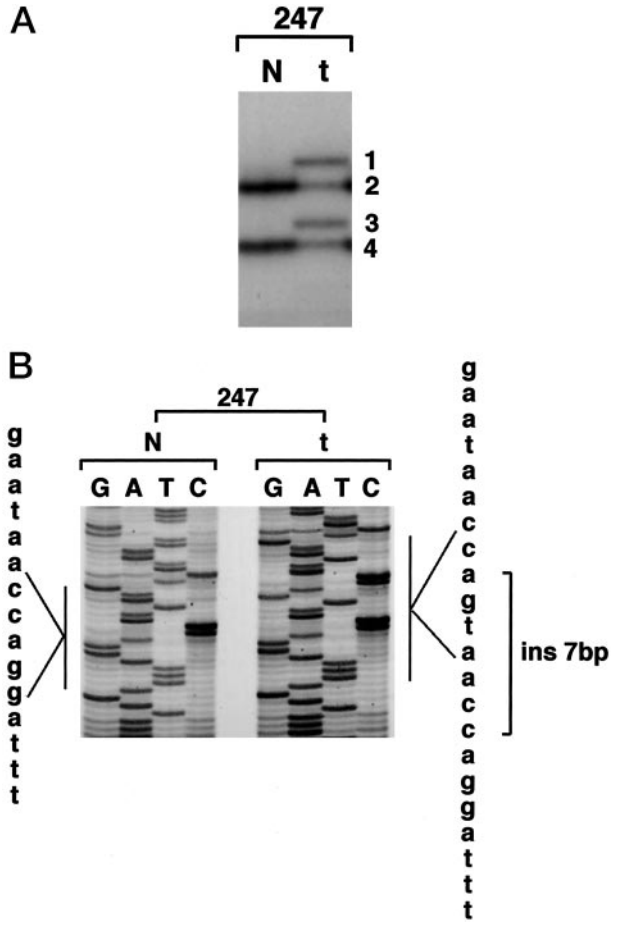

Fig. 1 Somatic mutation of *BRCA2* gene in case 247. *A,* SSCP gel, sequencing result shows that *bands 1* and *3* are strands of the mutant allele, *bands 2* and *4* are strands of the wild-type allele in the tumor. *B,* sequencing gel demonstrates somatic mutation with 7-bp (ccaatga) insertion after codon 25 of *BRCA2* resulting in a reading frameshift in the tumor.

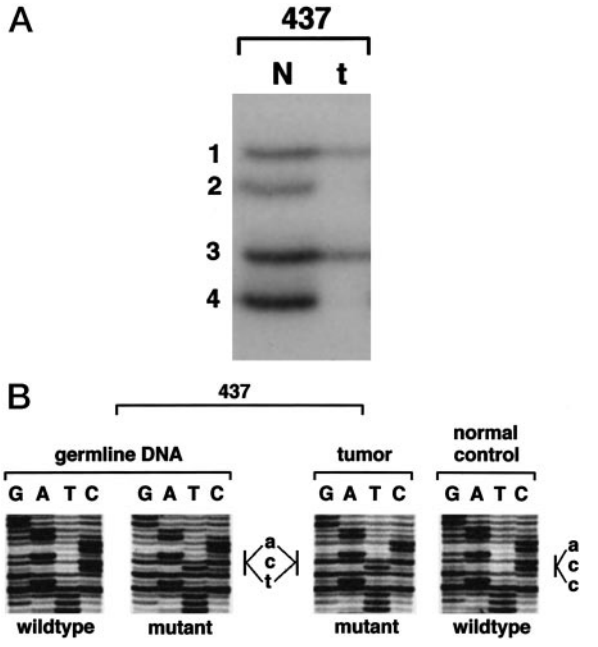

Fig. 2 Germline mutation and wild-type allelic loss in the tumor in case 437. *A,* SSCP gel shows an abnormal migration pattern in germ-line DNA; sequencing result demonstrates that *bands 1* and *3* are strands of the mutant allele and *bands 2* and *4* are strands of the wild-type allele in germ-line DNA. *B,* sequencing gel shows a missense mutation, C → T, resulting amino acid change of Pro → Ser at codon 3300 (P3300S).

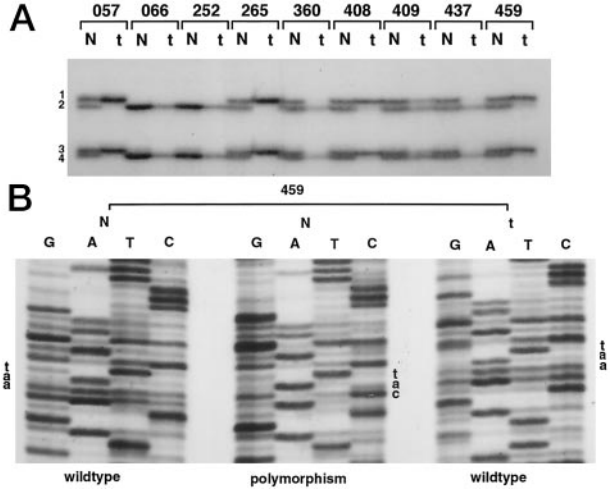

Fig. 3 Polymorphism and allelic loss at exon 11 (primer 11.7) of *BRCA2*. *A,* SSCP gel for 9 cases. *B,* sequencing demonstrates that *bands 1* and *3* are strands of the wild-type allele (His, H), and *bands 2* and *4* are strands of the polymorphic allele (Asn, N) at codon 372 (N372H). Genotype of case 459 is heterozygous and shows loss of the polymorphic allele in tumor. *N,* germ-line DNA; *t,* tumor DNA.

ever, none of the 56 tumors showed classic Knudsen two-hit inactivation with clear cut functionally inactivating mutations. Two cases showed LOH with missense mutations of unknown significance. Thus, we conclude that *BRCA2* is not the target of LOH on chromosome 13q. Because we did not evaluate *BRCA2* mRNA or protein levels, we do not know if function was altered in the cases with either biallelic or single allele changes. At present there are no compelling clinical or experimental data that we are aware of indicating that *BRCA2* haplo-insufficiency contributes to tumorigenesis (22). The three germ-line mutations we saw included one not reported previously, whereas the three polymorphisms we observed have all been reported before.[4] Distinguishing between mutations and polymorphisms in these patients is complicated by the fact that previous studies of these alterations in Chinese populations have not been reported. The overall significance of our findings is not known and may represent either biallelic inactivation of *BRCA2* in a small percentage of ESCC cases, or simply missense changes with no functional consequence. Whereas functional studies will be required to determine whether *BRCA2* has any role in ESCC, it is apparent from our results here that *BRCA2* is not frequently inactivated by the traditional two-hit mechanism. In summary, we showed for the first time that mutations in the *BRCA2* gene

do occur in ESCC patients but at low frequency. Moreover, the functional significance of these predominantly missense mutations remains to be determined. Evidence for classic biallelic inactivation was not seen. The putative target tumor suppressor gene corresponding to the high rate of chromosome 13q allelic loss remains unknown.

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